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Techniques for Preparing Plant Tissues for Optical and Scanning Electron Microscopy

by
Sarah E. Keates[1], Dale A. Draper[2] and Chris D. Hawkins[3]

[1] 249 Moran Crescent
Prince George, B.C.
V2M 6N4

[2] Research Branch
B.C. Ministry of Forests
31 Bastion Square
Victoria, B.C. V8W 3E7

[3] MacMillan Bloedel
65 Front Street
Nanaimo, B.C.
V9R 5H9

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Forestry Canada
Pacific Forestry Centre
506 West Burnside Road
Victoria, B.C. V8Z 1M5
(604) 388-0600

or

B.C. Ministry of Forests
Research Branch
31 Bastion Square
Victoria, B.C. V8W 3E7
(604) 387-6719

SUMMARY

This manual describes the procedures involved in preparing plant tissues in general and white spruce (*Picea glauca* [Moench] Voss) apical buds in particular for conventional light microscope and scanning electron microscope examination. In addition to presenting appropriate microtechniques, the formulation of processing solutions (Appendix 1), and the costs of associated materials (Appendix 2), the manual is designed to help the reader understand each procedure by providing background information and the reasons behind each operation. Once preliminary preparation (nursery operational level) and the preservation of tissues are completed, research material may be sent to laboratories specially equipped for further processing. This manual is not a compilation of techniques, as is available in other reference material. Instead, it focuses on training the practitioner, through the use of a case example, to master a set of basic procedures and principles necessary for preparing plant tissues.

PREFACE

The purpose of this manual is to provide information on dissecting, preserving, observing, and archiving conifer tissues. It describes a specific case example in which a basic schedule was used to process white spruce (*Picea glauca* [Moench] Voss) apical buds. The practitioner is introduced to the use of plant microtechnique for obtaining permanent reasearch records, in the form of slides and photomicrographs; and to basic theoretical and technical information that is needed to develop these records.

The manual is aimed at practitioners with little formal training or knowledge of microtechnique, be they in a fully equipped research installation or a minimally equipped quality control laboratory. The material presented is designed to aid reasearchers in estimating costs associated with microtechnique processing schedules, and to provide a case example for staff training.

The examination of conifer apical buds can be viewed as another means of seedling quality control, be it in the nursery (to test the chronological development of buds associated with cultural height control practices); the growth chamber (to provide records of bud damage associated with freezing or other stress tests); the laboratory (to quantify experimental treatments); or the field (to establish a history and prepare an autopsy for describing plantation responses to abiotic and biotic factors). This manual, therefore, addresses nurserymen, regional forest science officers, and research scientists.

Information was obtained from three major sources: textbooks, summarized from Johansen (4) and Berlyn and Miksche (1); unpublished instructional material, Biology 344 (Plant Microtechnique) Laboratory Manual (John Owens, compiler). Biology Dept., University of Victoria, Victoria, B.C.; and personal communication/technical consultation with Joanne E. MacDonald, Biology Dept., University of Victoria, Victoria, B.C.

This manual is not intended to summarize all available technical/theoretical plant microtechnique information; nor is the information presented in the case example intended for use with material or processing schedules other than those specifically mentioned. Further theoretical/technical information for designing schedules for processing other classes of plant material, or to satisfy research demands other than those outlined in this paper, can be obtained from the literature cited in this manual, and from associated resource materials.

Along with the experimental photo-record also being produced as part of FRDA Project 1.36,[1] the manual should allow the inexperienced practitioner to reproduce the schedule outlined and to obtain similar research material.

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[1] C.D.B Hawkins and D.A. Draper. 1988. The use of photoperiodic cultural treatments to induce dormancy and terminal bud formation of spruce in northern nurseries. B.C. Ministry of Forests. Working Plan. FRDA Project 1.36. Unpublished.

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1 INTRODUCTION

Structure and function are the fundamentals of plant science (1). Producers of conifer seedlings, by assessing the structure and awareness of associated function, can critically evaluate cultural techniques. Evaluation may lead to modifications to culture in a single growing season, or to improved understanding over several years. Studying material at the tissue level is central to understanding these fundamentals and provides the basic information required to produce plant material for specific purposes.

Microtechnique as defined by Sass (5) consists of three components: 1) preparation of specimens for microscopic examination; 2) critical study and interpretation of prepared material with the use of the microscope and associated equipment; and 3) production of permanent research records in the form of graphical and numerical information (1).

The technical information presented in this paper is divided into a series of logical steps. Availability of equipment, and nursery operational or research demands should be considered to determine the appropriate level of investigation for each project. The initial steps of dissecting and fixing material can be done with minimal equipment and labour. Further processing involves specialized equipment and increased labour and is easily transferrable to laboratories designed to complete this and produce graphical and numerical information.

With optical and scanning electron microscopy incorporated into experimental analysis, it is possible to obtain structural and functional information. Therefore, mastering an effective set of techniques for studying cells and tissues is intrinsic to investigating plant developmental processes.

2 PROCESSING AND EMBEDDING APICAL BUDS FOR LIGHT MICROSCOPY

2.1 Introduction

The following section summarizes the processing schedule used to trace the development of white spruce (*Picea glauca* [Moench] Voss) terminal buds induced with photoperiodic treatments (blackout) as outlined in FRDA Project 1.36.[2] Samples of buds were taken at intervals of approximately 1-2 weeks to provide a detailed record of progressive changes in apical bud morphology. Median longitudinal sections will act as a permanent record of the development of buds in each treatment, as well as providing photomicrograph and numerical information to augment additional experimental measurements made over the growing season.

2.2 Dissection

2.2.1 Theory

Dissection is the first step in preparing samples for light microscope examination. The purpose of dissection is to:

1. obtain workable sections of material of appropriate size for optimum tissue fixation;
2. obtain sections which are representative of the tissue at time of sample and which contain morphological features needed for developmental analysis.

The quality of initial tissue dissection determines the quality of the final microtomed sections. The initial plane of sectioning depends on the initial plane of dissection. In addition, the appearance of tissues in the final sections depends on accurate dissection. Tissues desiccated or crushed during dissection are useless in further processing. Improper dissection may also prevent proper fixation of tissues which also affects sectioning.

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[2] C.D.B Hawkins and D.A. Draper. 1988. The use of photoperiodic cultural treatments to induce dormancy and terminal bud formation of spruce in northern nurseries. B.C. Ministry of Forests. Working Plan. FRDA Project 1.36. Unpublished.

2.2.2 Method

- To prepare for bud dissection, remove all needles obscuring the shoot apex by stripping them away from the stem with extra fine point forceps. While viewing the shoot apex under a stereomicroscope at 65-100 times magnification, carefully remove the needles directly surrounding the developing bud by grasping them at their bases and peeling rather than snapping them away from the stem tissue. Next remove the bud scales enclosing the vegetative shoot tip. This procedure makes the shoots easier to dissect, fix, and section. Although processing is more difficult when bud scale tissue is retained, it is possible to do if initial sectioning is done before fixation. Initial sections allow fixatives to penetrate the bud tissues evely, and section tearing can be avoided.
    - Dissection must be executed quickly and carefully to prevent mechanical damage and desiccation in the stem tissue. This is particularly important when scot seedling tissues are dissected.
- Using a clean, double-edged razor blade, make a cross-sectional cut approximately 3-4 mm below the base of the bud scales to remove the bud from the shoot.
    - Place the bud in an upright position on the stage plate of the dissecting microscope and make two longitudinal sections parallel to the desired plane of sectioning (Figure 1).

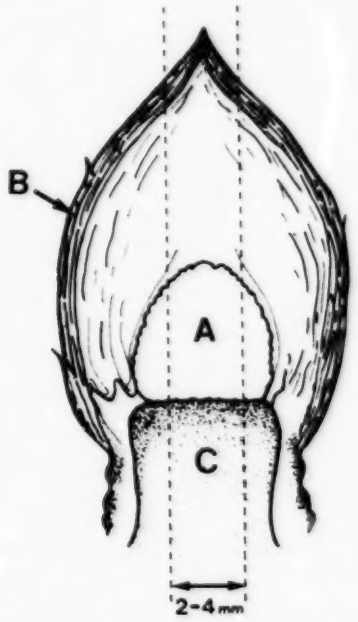

FIGURE 1. Dissection scheme for preparing white spruce (*Picea glauca* [Moench] Voss) apical bud longitudinal sections for embedding and microtoming: A, vegetative shoot within terminal vegetative bud; B, bud scales; C, stem tissue (dashed line = plane of sectioning).

- Samples should be between 2 and 4 mm thick to allow for complete and rapid tissue fixation.
- Several important procedures should be followed to avoid damage to tissues during dissection:
    - Sections should be made with an even slicing motion rather than a downward chopping into the tissue, which creates greater pressure and may crush cells on either side of the blade.
    - Clean razor blades between each section with a suitable solvent such as 70% ethanol, and change blades frequently. Tissue damage and uneven sections are easily caused by using dull or resin-coated blades.

- When sectioning, stabilize the bud by resting it against the nail of an index finger rather than using pressure to hold it in place. Added pressure on the surface of the bud during dissection may result in tissue disruption.

2.3 Fixation

2.3.1 Theory

Fixation determines the degree to which the prepared tissues represent the material at the time of sampling (4). The main objective of fixation is to terminate life processes within tissues and preserve material in a natural state (1). The fixation process generally, and the fixative specifically, must retain the material in an undistorted state that will hold up during all subsequent steps (1). Unlike unfixed tissue (which is susceptible to desiccation, shrinkage and swelling, autolysis, pH effects, and temperature), fixed tissue is stable and readily suited to the steps described below (1). Fixation facilitates the production of permanent research records in the form of microscope slides and photomicrographs (1).

Formalin acetic alcohol (FAA) is one of the most commonly used plant fixatives (4). Alcohol percent per volume should not exceed 70% because of its shrinking effect on tissue (1). Just as amounts of formalin and alcohol in this fixative can be altered to reduce shrinkage and precipitation of proteins, similarly the amount of acetic acid can be adjusted to reduce swelling of the protoplasm.

For many fixation needs FAA is an attractive choice because it penetrates rapidly, leaving less time for chemical denaturation as it travels through the tissue (4). It is also extremely stable, and, therefore, material can be stored in it for up to several years (1). Although rapid hardening is advantageous in preserving tissues in their natural state, care must be used in deciding on an appropriate fixation time (4). For instance, sections of thin leaf are fixed in as little as 12 hours, whereas woody stems should be fixed up to 1 week before embedding (4). Longer fixation may cause vascular tissue to become much harder than protoplasmic structures, making sectioning difficult (1).

2.3.2 Method

- Immediately following dissection, samples should be transferred to an appropriate fixative such as FAA (see Appendix 1, part A, section 1.0 for formulation, and Appendix 2, part A, section 1.0 for chemical costs). Extended exposure of specimens to air may lead to desiccation and tissue destruction.
    - Fixative should be contained in unreactive glass vials. Fill the vials approximately two-thirds full of fixative (ideally, use between 5 and 10 times the volume of fixative to volume of buds). Store no more than six to nine sections per vial.
    - After the material is in the fixing solution, aspirate the sample at 15 lb pressure for 1-5 minutes to withdraw air from the intercellular spaces of the tissue. Do not aspirate longer than the listed time, to prevent bubbling the fixative.
    - Samples should be fixed an average of 12-24 hours before washing and dehydration procedures.
- Prepare a label, using soft lead pencil, that shows the specimen number and date, species, plant part fixed, type of section, and fixative used. This label remains with the samples throughout processing and is embedded with the specimens in the paraffin casting block. Record casting block identification numbers and details on the corresponding sample in a notebook to have permanent record of the material contained in each casting block.

2.4 Washing and Dehydration

2.4.1 Theory

It is normally necessary to remove all traces of the fixing solution from the specimens before tissue dehydration and further processing because fixatives may:

1. inhibit staining;
2. cause formation of unwanted precipitates; or
3. damage the tissues as a result of residual fixing activity (4).

Virtually all aqueous fixatives (such as Nawaschin's and Randolph's solutions) are washed out with water. Alcoholic solutions (such as FAA or Carnoy's fluid) are washed out, starting with alcohol of the same concentration as contained in the fixative (4).

To introduce washed tissues into the dehydration process gradually, the specimens should be run through a series of alcohol solutions (of progressively increasing concentration) until the initial concentration of alcohol in the chosen dehydration series is reached (4). Improper transition from washing solution to dehydrating agent increases the risk of tissue plasmolysis, leading to visible distortion of cells in resulting sections (1). For example, specimens washed in water must be run through 15, 30, and 50% alcohol before they are introduced to a dehydration series in which the first solution contains 50% alcohol (4). Similarly, samples washed in alcohol should be placed into the first solution in the dehydration series which is of corresponding concentration. This prevents tissue lysis (4).

Before fixed specimens are embedded in a wax medium, all traces of water must be removed from them to ensure that the wax will penetrate all internal and external surfaces of the tissue (1). The dehydration process is designed to treat tissues with solutions containing progressively higher concentrations of the dehydrating agent to water (1). Two methods can be used in tissue dehydration.

1. Nonsolvents of paraffin, such as ethanol, can be used as the primary dehydrating agent. It is necessary to ensure complete dehydration before the tissues are cleared in a solvent or paraffin, such as xylene or chloroform.
2. Solvents of paraffin, such as tertiary butyl alcohol (TBA), can be used as dehydrating agents. Material can be transferred into xylene or chloroform at a point when complete dehydration has not yet been reached (4).

The TBA dehydration series outlined by Johansen (4) is widely used and produces wholly satisfactory results in many applications (1). Although TBA is expensive and extremely flammable, in addition to requiring storage above room temperature, it is a good choice because it does not produce the hardening associated with using ethyl and propyl alcohol, acetone, xylene, or even normal butyl alcohol (1). The use of TBA as a dehydrating agent has dramatically broadened the range of applicability of the paraffin method (1).

2.4.2 Method

- The next step in the processing schedule is to wash the fixative out of the tissues in preparation for dehydration.
  - The washing solution used represents the base ingredient in the fixative. In the case of 50% FAA, use a washing solution of 50% ethanol (the concentration of alcohol contained in the fixative). In the case of other fixatives such as Nawaschin's and Randolph's solutions which have a water base, wash the specimens in running tap water.
  - Following thorough washing, samples should be run through a dehydration series to remove water from the tissues.
- The washed sample should then be run through the Johansen dehydration series which uses TBA as the primary dehydrating agent.
  - The schedule presented in Table 1 outlines an accepted dehydration procedure (after Johansen [4]). (For specifics on preparation of Johansen dehydration series solutions, see Appendix 1, part A , section 2.0, and Appendix 2, part A , section 2.0 for the cost of chemicals.)
  - Throughout the series, unless otherwise stated, use just enough solution to cover the plant material. Times listed in Table 1 represent **minimum** times in each solution.

- Make changes rapidly and according to the appropriate time schedule by decanting the previous solution from the vial — ensuring that samples remain intact — and immediately replacing it with the next solution in the series. Cork the vials after each change to prevent evaporation of the solutions.
- Johansen number two and number six are stable solutions in the series. On day one, run samples up to Johansen number two and leave overnight. The following day, take samples through to Johansen number six (100% TBA) and store them at ≥ 26°C, to prevent crystallization of the solution. Specimens may remain in Johansen number six until preparations are completed for infiltration and embedding.
- Check the vials at regular intervals to ensure that they are sealed and that no evaporation has occurred. If evaporation is evident, top up the solution in the vial and replace the cork.
- Leaving samples in solutions other than Johansen number two and six for extended periods is not advisable.

TABLE 1. Johansen dehydration series and embedding schedule for material processed using the paraffin method[1]

Day	Time	Change from	Change to	Time in new solution	
1	08:00	50% FAA	50% ethanol		1h
	09:00	50% ethanol	50% ethanol		1h
	10:00	50% ethanol	50% ethanol		1h
	11:00	50% ethanol	50% ethanol		1h
	12:00	50% ethanol	Johansen #1		2h
	14:00	Johansen #1	Johansen #2		2h or ON[@]
2	08:00	Johansen #2	Johansen #3		2h
	10:00	Johansen #3	Johansen #4		2h
	12:00	Johansen #4	Johansen #5		2h
	14:00	Johansen #5	Johansen #6	store @ ≥26°C	2h or ON
3	08:00	Johansen #6	Johansen #6	*	1-2h
	10:00	Johansen #6	Johansen #6	*	1-2h
	12:00	Johansen #6	Johansen #6	*	1-2h
	14:00	Johansen #6	Johansen #7	*	1-2h
	16:00	Johansen #7	#7/paraffin*		1-4h @ ≥26°C then ON @ 65°C
4	08:00	#7/paraffin	Tissue Prep®	in 65°C oven	1-2h
	10:00	Tissue Prep®	Tissue Prep®	*	1-2h
	12:00	Tissue Prep®	Tissue Prep®	*	1-2h
	14:00	Tissue Prep®	Tissue Prep®	in 65°C oven	ON
5	08:00	Tissue Prep®	Tissue Prep®+	*	1-2h
	10:00	-EMBED-			

1 Biology 344 (Plant Microtechnique) Laboratory Manual (John Owens, compiler), Biology Dept., University of Victoria, Victoria, B.C. Unpublished.

@ ON = overnight.

* Pour samples in Johansen #7 into vial 2/3 full of hardened Tissue Prep®.

+ Fill vial completely with Tissue Prep® on this change.

2.5 Infiltration and Embedding

2.5.1 Theory

To thin-section tissues for microscopic analysis, it is necessary to cast the specimens in a solid matrix both to support the tissues against the impact of the blade and to maintain physical features in proper relation to each other (1). Paraffin wax of known melting point, appropriate hardness, even texture, and purity is an excellent medium for creating such a matrix (1). The process of infiltrating dehydrated material with paraffin must be gradual to ensure that the matrix adheres to all external and internal surfaces and completely fills all tissue cavities (1).

In the Johansen dehydration series, gradual transfer from the dehydrating agent (TBA) to paraffin is facilitated if the specimens are first placed in a solution of equal parts paraffin oil to TBA (4). Paraffin oil aids gradual infusion of the wax and prevents heat damage to tissues (4). After this, the samples should be transferred to a vial partially filled with solidified paraffin and covered with the solution of paraffin oil and TBA (4). The vial should then be placed in a ventilated oven in which the paraffin gradually melts and the alcohol evaporates from the mixture (4). As the samples slowly sink and finally reach the bottom of the vial, they are gradually infiltrated and saturated with the melted paraffin (4).

All TBA and paraffin oil must be removed from samples before they are embedded. This means making several changes of melted paraffin over a 24-hour period (4). The final change of paraffin should not produce the odour of TBA, which is evidence of incomplete solvent evaporation (4). Once material is in the ovens, it should not be left for periods of time longer than those stated in the schedule (4). If paraffin remains in a melted state for an extended time, fractions with low melting point may evaporate. This yields unsatisfactory paraffin ribbons during sectioning (1).

It is safe to proceed with embedding once it has been established that all alcohol and paraffin oil has been removed from the tissue samples (4). Embedding consists of pouring the samples and the melted embedding medium into casting receptacles, placing the specimens in an orderly arrangement, and then cooling the medium as quickly as possible (4). Although use of a warming plate is recommended during this procedure to prevent premature cooling of the lower surface of the wax before set-up, the paraffin must not be overheated (1). Probes used to arrange the material should be heated to avoid the formation of lines in the block caused by partial solidification of the wax in the instrument's path (1).

Immediately after specimens are arranged, the casting block must be cooled. Paraffin may crystallize if it is allowed to cool too slowly (4). Blocks showing fluffy white areas, indicative of crystallized paraffin, cannot be microtomed because the paraffin no longer has an even texture (4). Embedded samples showing crystallization must be recast to salvage the material (4).

2.5.2 Method

- The next procedure in preparing samples for casting is to gradually infiltrate the dehydrated plant tissues with paraffin wax of known purity and melting point. Tissue Prep® is an effective embedding medium (a cost analysis of infiltration/embedding materials is given in Appendix 2, part A , section 3.0, and part C, section 1.0).
    - The initial step in the transition from dehydrating agent to paraffin is to soak the samples for 1-2 hours in Johansen number seven, a TBA-paraffin oil solution (see Table 1). Harder materials, such as woody stems, must be soaked longer. Following this step, pour the samples and the Johansen number seven into a vial one- to two-thirds full of hardened paraffin (i.e., Tissue Prep®). Ensure that there is just enough Johansen number seven present to cover the samples.
    - Following a period of 1-4 hours at room temperature, place the uncorked vials in a 65°C oven for 3-4 hours, or until the paraffin is completely melted. Samples may be left overnight in the oven, but no longer.
    - The following day, discard the Johansen number seven paraffin mixture and add enough fresh Tissue Prep® to cover the samples.

- Make three additional changes of Tissue Prep® over the next 24 hours.
- The next day, make a final change to new Tissue Prep®. Completely fill the vial with this change. After 2 hours in the oven, the samples are ready to embed.

- Following infiltration, tissues must be embedded in a solid paraffin matrix. Embed the sectioned buds the day after the final step in the infiltration process. It is not recommended to leave vials in the oven longer than the listed times. Care should be taken to ensure that the number of samples to be infiltrated and then embedded the next day is not overestimated.
  - Samples may be cast in specially designed trays, although manilla trays or "boats" are inexpensive and do the same job (1). A warming plate with both a heated and an unheated section should be used during the embedding process. (For a listing of an acceptable brand and its corresponding cost, see Appendix 2, part C, section 1.0.)
    - Remove samples from the oven and pour the contents of the vial, containing the material in the most recent change of Tissue Prep®, into a casting boat. It may be necessary to add additional melted Tissue Prep® to cover the samples fully.
  - Using a heated probe, separate and arrange the samples into evenly spaced rows while maintaining the correct orientation of the apical buds for sectioning. Leave enough space between sections to allow for the block to be cut into separate specimen blocks, each containing an individual bud section. Place the block identification tag face-side down in the corner of the boat (see Figure 2). Work quickly to prevent overheating the paraffin.

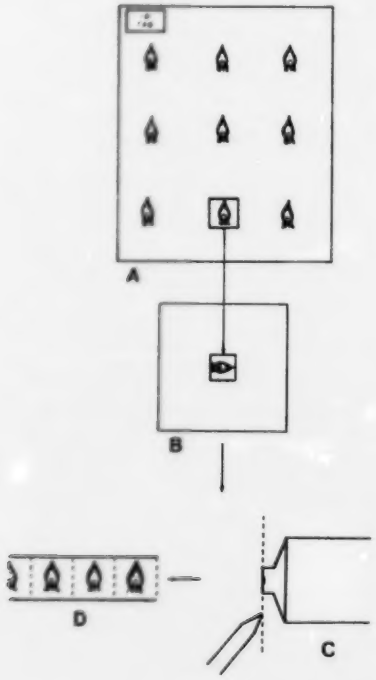

FIGURE 2. Arrangement of specimens in the casting block and on the object block to achieve the desired plane of sectioning: A, arrangement of nine sections and identification tag in casting block; B, specimen block mounted on the object block; C, orientation of the specimen on the object block to the microtome blade (dashed line = plane of sectioning); D, completed paraffin ribbon with longitudinal sections joined side by side (after Berlyn and Miksche [1]).

- While correcting the set-up of the material, slide the casting boat to the cool portion of the plate. Proper set-up must be achieved by the time the boat reaches the unheated portion of the plate, because it will begin to solidify.
- When the lower layer of the paraffin solidifies and appears opaque, transfer the boat to a sink filled with cold water and float the boat on the surface. It is important that water does not contact the surface of the block until a solid top layer has formed.
  - To speed up the solidification of the top layer of the block, gently blow on the surface of the paraffin.
- When the upper surface of the block has solidified and appears opaque-white, immerse the boat in the water for 15-30 minutes. Remove the boat from the water and blot the surfaces dry.
- The paper boat can be peeled off the casting block 5 minutes after it is removed from the sink, or, if it is to be reused, wait until the boat is completely dry before peeling it off the block.

2.6 Storage of Paraffin Blocks Before Sectioning

The paraffin casting blocks containing the identification tag and samples should be stored flat in a cool, dark area. To expose sample information and facilitate filing, it may be necessary to melt the left hand corner of the paraffin block above the identification tag. Separate blocks with cardboard dividers or place them individually in labelled envelopes to prevent them from adhering to each other. Blocks stored in this manner will remain stable for an indefinite period of time or until they are microtomed.

2.7 Preparing Embedded Material for Sectioning

2.7.1 Theory

Tissues processed in a dehydration series are often hardened and must be softened prior to microtoming. Gifford's solution, containing glacial acetic acid (see Appendix 1, part A , section 3.0 for formulation; Appendix 2, part A, section 4.0 for costs), is commonly used to soften tissues so that they may be sectioned more easily.[3]

2.7.2 Method

- Cut the casting block into separate specimen blocks to prepare for microtoming.
  - Using a heated putty knife, melt a grid over the casting block to separate it into individual specimen blocks. Cut the sections of the block containing the specimens apart. The individual pieces will vary in size and should be trimmed before they are mounted on object blocks. A margin of approximately 5-7 mm around each bud section is adequate. Specimen blocks that are too small to be mounted on object blocks can not be corrected by any means other than re-embedding.
  - The identification tag is similarly cut out of the block and, if necessary, the paraffin is melted slightly to expose the information on the tag.
- Most tissues must be softened before they can be microtomed. Place the specimen blocks, along with the embedded identification tag, in plastic vials and add enough Gifford's solution to fully cover the material.
  - Date and cap the vials before placing them in a 38°C oven. The samples should remain in the softening solution for a minimum of 1 day. Exact time sufficient for perfect softening, however, can only be determined by trial and error.
- Following softening, trim the specimen blocks down using razor blades until the material is surrounded by a 3-mm margin of paraffin on all sides of the block except the bottom, where a

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3 Biology 344 (Plant Microtechnique) Laboratory Manual (John Owens, compiler). Biology Dept., University of Victoria, Victoria, B.C. Unpublished.

5-mm margin should be left. This procedure will require some practice to perfect. The specimen blocks are now ready for mounting.

- Once the desired size is achieved, the specimen blocks should be melted onto plastic or wooden object blocks. Melt the residues of paraffin on the object block by using the side of a heated knife. Slightly heat the specimen block on its lower surface and press the blocks together ensuring that the paraffin block is straight on the object block. The grid surface found on many object blocks serves as a good reference for straightening the mount.
  - Ensure that blocks are mounted in the correct orientation to produce the desired section and as many sections per coverslip as required (see Figure 2).
- Level the sectioning surface of the paraffin block using a heated razor blade or the side of a heated putty knife. An uneven block surface will result in the formation of sections of variable thickness during sectioning because the block will not sit squarely to the microtome blade.
- To cool the block and prevent compression of the paraffin during sectioning, place completed object blocks in the freezer compartment of a fridge (at approximately -10°C) for 10-15 minutes, or use a dry ice bath.

2.8 Sectioning

2.8.1 Theory

Plant material processed using the paraffin method is generally sectioned with a rotary microtome (1). The blade of a rotary microtome remains stationary and the specimen block is moved up and down past the cutting edge, producing sections by wedge action (1). Following each cut, an automatic carrier advances the block forward to a specific point, producing a continuous ribbon of sections, each of identical thickness and in serial order (1). Section thickness is controlled with such precision that it is possible to determine if the material is the appropriate number of cells thick to present all morphological features clearly, yet still maintain the continuity of cell layers within the tissue (1). The serial order of the sections can be maintained through further processing of the tissue, creating a permanent record of the complete external and internal structure of the sample (1).

Sectioning material is the most difficult step in the paraffin method because of the many variables involved in obtaining satisfactory sections (1).

Quality of the paraffin directly influences the quality of the resulting sections (1). The paraffin should have a smooth texture free from bubbles, crystals and debris (4). It should also be of appropriate hardness to support the tissues at the desired sectioning thickness, and at the room temperature at which the cutting is done (1).

Material must be completely infiltrated with paraffin before it is microtomed (1). Crumbling within tissue may indicate incomplete penetration of wax during infiltration or excessive tissue hardness (1). If entire sections break out of the paraffin ribbon, external surfaces may not have adhered to the paraffin. Improper infiltration is often caused by incomplete tissue dehydration or too rapid an infiltration of paraffin (1). To correct these problems, tissue must be reinfiltrated and recast (1).

The orientation of mounted specimens must be considered before they are sectioned. Samples should be laterally and vertically centred in the sectioning block and the block should be trimmed so that subsequent sections will be close enough together in the ribbon (1). The face of the block should be parallel to the blade and the blade should be at right angles to the vertical movement of the block (1). The angle of the blade to the block must be determined by trial and error. It depends on the sample, blade, and microtome (1).

The embedded sample should be firmly affixed to the mounting block. In addition, the mounting block, knife, and knife carrier must be clamped firmly in place to produce even sections of identical thickness (1). If blocks are mounted too high, they may fall off the carrier or vibrate, resulting in uneven sections (1). Because uneven section thickness is often not apparent until after staining, considerable amounts of time may be wasted. Therefore, ensuring rigidity of the mount before microtoming is extremely important (1).

The temperature of the paraffin block and the surrounding environment also influence sectioning (1). If the temperature of the block or room is too high, sections may compress on impact with the microtome blade (1). Conversely, if the temperature is too low, sections may curl on the blade and fail to form a continuous ribbon (1). To prevent compression of sections, which is the most commonly encountered temperature-induced problem, the block and/or blade should be cooled before the material is microtomed (J.E. MacDonald, pers. comm., June, 1988, University of Victoria, Victoria, B.C.). Cooling can be accomplished using dry ice or by placing the mounted block in a freezer compartment (at approximately -10°C) for 10-15 minutes (J.E. MacDonald, pers. comm., June, 1988, University of Victoria, Victoria, B.C.).

Although many plant tissues are easily cut during dissection, they may become hardened following dehydration and embedding (4). The hardness of embedded material directly influences the quality of resulting sections (1). Sections which contain tears indicative of excessive tissue hardness must be softened before they can be microtomed successfully (4). Softening involves exposing embedded tissues to warm water or a specially formulated softening agent such as Gifford's solution or hydrofluoric acid (4). The softening process is based on the assumption that embedded tissues retain their ability to take up liquid (4). This procedure may be done before sectioning (e.g., with woody stem material), or after initial sections are made and tearing is evident (4). The rate of penetration of the softening agent can be gauged by the increase in opacity of the paraffin surrounding the material. However, softening time for a given sample often varies greatly (4). The extent of softening should be observed every 12 hours to prevent oversoftening of the tissues (1). Specimens that are softened longer than necessary become macerated and must be discarded.

The operation of a rotary microtome is best learned through careful observation of the techniques of an experienced user (4). For written descriptions of microtome use and operating principles, consult the manufacturer's manual or comprehensive microtechnique texts such as Berlyn and Miksche (1:61-62) and Johansen (4:140-146).

2.8.2 Method

- To prepare for microtoming the embedded specimen, adjust microtome sectioning thickness to 10 µm (for a cost estimate of a standard rotary microtome and suggested brand, consult Appendix 2, part C, section 2.0).
  - Place the object block in the holder (ensuring that the wheel lock is on) and move the block to within 2-4 mm of the blade.
    - View the surface of the block and the angle of the blade from all sides to determine if the block is on plane with the blade and if the blade is at an appropriate angle for sectioning.
    - Make adjustments to the blade and block to produce the desired set-up.
  - Move the block forward until it almost contacts the blade before beginning the sectioning process.
    - Initiate sectioning at one end of the blade and work toward the opposite end.
    - Gently suspend the resulting paraffin ribbon with a moistened paint brush.
      - If the ribbon curls, trim the block down to make it level and repeat the alignment procedure.
      - If the ribbon breaks repeatedly, clean the surface of the blade to remove all debris. This should help to free sections from the blade.
- Once the embedded bud sample is reached, carefully observe the ribbon for sections showing streaks or holes.
  - Streaks in the sectioned paraffin may indicate one of two problems:
    - There may be a nick in the blade, causing tears in the sections. To correct this problem, move the blade along and resume sectioning.
    - The embedded material may be excessively hard causing tears in the section. To correct this, remove the specimen block from the object block and return it to the softening

solution. Remember that the tissue, already sectioned, is in direct contact with the softening solution. Because of this, monitor further softening carefully and attempt sections at regular intervals (e.g., every 12 hours) to prevent oversoftening the specimens. Macerated tissue cannot be salvaged.

- Holes in tissue sections indicate the presence of air bubbles in tissue or in the casting block. Re-embedding the samples may correct this problem.

- Periodically view acceptable ribbons under a light microscope to determine when the desired median longitudinal section of the apical bud has been reached. Judging the point at which the median longitudinal section has been reached is difficult but becomes more obvious with increased experience in microtoming material.

- Once the portion of the ribbon containing median longitudinal sections of the apical bud has been reached, cut the ribbon and place it glossy-side down on black construction paper. Do not place the paraffin ribbon where it cannot be seen readily, or on a smooth surface to which it will adhere.

2.9 Mounting the Ribbon

2.9.1 Theory

Satisfactory paraffin sections, in the form of a ribbon, are fastened to microscope slides with adhesive before staining is done (1). To ensure proper adhesion, several procedures must be followed.

- Slides must be cleaned with a suitable solvent (70% ethanol) to remove residues that could interfere with adhesion (1).

- A strong adhesive must be used. Haupt's adhesive is considered by researchers to be unexcelled for mounting sections (4).

- Heat used to flatten sections must be sufficient to remove all wrinkles in the paraffin without melting it (4).

- Slides must be dried completely before the sections are stained (1). This ensures that the section is affixed firmly to the slide and that the adhesive is hardened completely, making it insoluble in the reagents used in staining (1).

Dried slides can be stored, possibly indefinitely, until the appropriate staining schedule is carried out (1).

2.9.2 Method

- Fill a staining tray with 70% ethanol and soak several microscope slides to remove possible residues. Dry slides thoroughly.

  - Coat the cleaned slide with an **extremely** thin film of Haupt's adhesive (see Appendix 1, part A, section 4.0 for formulation, and Appendix 2, part A, section 5.0 for cost of chemicals), and place it on a slide warmer until it is dry.

  - Flood the slide with a 4% solution of formalin.

- Separate a series or an individual section from an acceptable paraffin ribbon using a single-edged razor blade. The relationship between the size and characteristics of the sectioned material and the size of the cover glass to be used should be considered before sections are mounted.

  - Transfer the selected section(s) to the prepared slide on a 43°C warming plate, and float them lower, glossy-side down on the formalin (for cost of slide warmer, see Appendix 2, part C, section 3.0).

  - Arrange section(s) in an orderly manner slightly right of centre to allow for a label to be placed in the left-hand corner of the slide.

- Leave the slides on the warming tray, checking the set-up, until the section(s) have adhered to the slide in their proper orientation. Ensure that the warming plate temperature is not above 43°C so that the section(s) may be set up properly and the paraffin does not melt.
- Drain excess formalin from the slides and move them to a 38°C oven for drying.
- Mounted sections should be dried a minimum of 12 hours before they are stained.

2.10 Staining

2.10.1 Theory

Differential staining of tissues is based on specific affinities between certain dyes and particular cellular structures (1). Choosing an appropriate staining regime for sectioned material depends on the characteristics of the plant material and on the specific research demands. Two of the more common staining combinations for botanical tissues include:

1. iron hematoxylin with or without a suitable counterstain; and
2. safranin/fast green (4).

There are many acceptable staining combinations that impart specific character to sectioned plant material. Berlyn and Miksche (1) have reviewed several of the most commonly used stains. To stain conifer bud apical longitudinal sections, a basic anatomy stain composed of the contrasting stains, Safranin O and Heidenhain's hematoxylin, is useful (see Appendix 1, part A, section 5.0 for formulation, and Appendix 2, part A, section 6.0 for chemical costs) (J.E. MacDonald, pers. comm., June 1988, University of Victoria, Victoria, B.C.).

Heidenhain's iron hematoxylin and safranin O is a useful staining combination for most mature plant tissues (4). Hematoxylin, a natural dye derived from logwood (*Hematoxylin campechianum* L.) has little or no natural affinity for tissues and is used in conjunction with a mordant such as ferric chloride or ferric alum (4). The colour effects of hematoxylin vary with the character of the medium it is dissolved in, and the type of after-treatment (4). In acid solution, hematoxylin stains red; in alkaline solution, it stains blue (4). Hematoxylin shows an affinity for chromosomes, mitochondria, plastids, and unlignified cell walls, staining them blue-black to black in alkaline solution (4).

Safranin O, a basic stain, is a member of the coal tar dye group. It imparts a red stain to lignified, cutinized, and suberized cell walls, as well as centrosomes, chromosomes, and nucleoli (4). Safranin is known to overstain and for this reason excess stain should be washed away with water to prevent precipitates from depositing in the tissues (4).

In the completed stained section, look for possible artifacts, such as brown precipitate in parenchyma caused by old hematoxylin, and also for proper colour and differentiation (1). Improperly stained material is easily restained by reversing the schedule and correcting the stain at the appropriate step(s) (4).

Staining regimes and their appropriate uses are outlined in microtechnique texts such as Berlyn and Miksche (1:85-108), and Johansen (4:49-94, 151-154). Further information is also available in staining procedure texts such as Clark (2) and Conn (3).

2.10.2 Method

- An outline of the Safranin-Hematoxylin staining schedule is presented in Table 2.
- All preparatory and staining solutions should be used in the fume hood in appropriate staining vessels such as staining trays (Chemonics Scientific Ltd.). These trays allow even contact between all slides in the set and the staining solution in which they are immersed, and will prevent the slides from adhering to each other (1) (see Appendix 2, part B, for cost of trays).
  - The first step in the staining procedure is to remove paraffin from the tissue sections and prepare them for stain infusion. This is done by washing the slides in Hemo-De®.
    - Presence of a white emulsion in the Hemo-De® rinse indicates improper drying of the slide. This is most often the result of using excessive amounts of adhesive during mounting.

- Rinse slides showing emulsion in 100% ethanol.
    - If 100% ethanol fails to remove the emulsion, discard the sections.
- Hemo-De® is washed from the slides using 100% ethanol.
    - In some cases, a second rinse of Hemo-De® may be necessary because of a build-up of paraffin in the first rinse.
- When all traces of Hemo-De® have been removed from the slides, run the slides into a hydration series of 100, 95, and 50% ethanol and finally into the solvent of the staining solution, in this case, tap water.
- Place the slides in the first staining solution, safrinin, for a period of 30-60 minutes or longer.
    - Remove excess safrinin from the slides with tap water and immerse them in a 4% aqueous ferric chloride mordant (see Appendix 1, part A, section 5.0 for formulation, and Appendix 2, part A, section 6.0 for associated cost).

TABLE 2. Safrinin O - hematoxylin (Heidenhain's) staining schedule for plant tissue processed using the paraffin method[1]

Step	Reagent used	Time in reagent	Specific instructions
1	Hemo-De®	15 min	ensure no precipitate
2	100% ethanol	5 min	
3	95% ethanol	5 min	
4	50% ethanol	5 min	
5	distilled water	5 min	
6	1% (aq) safrinin O	30-60 min	may require longer
7	distilled water	4 X 5 min	
8	4% (aq) ferric chloride	20 min	
9	distilled water	4 X 2 min	
10	hematoxylin	8-10 min*	8 min if fresh tissue
11	tap water	4 X 5 min	water should turn blue
12	4% (aq) ferric chloride	3-5 sec	or destain longer
13	tap water	4 X 5 min	change #4 add squirt NaOH
14	50% ethanol	3 min	maximum time*
15	95% ethanol	3 min	maximum time*
16	100% ethanol	3 min	maximum time*
17	Hemo-De®		long enough to clear; judge slides then mount

[1] Biology 344 (Plant Microtechnique) Laboratory Manual (John Owens,compiler). Biology Dept., University of Victoria, Victoria, B.C. Unpublished.

* Amount of time in hematoxylin is extremely variable depending on the state of the tissue (fresh or not). It is very difficult to judge the colour until slides have been cleared in Hemo-De®.

- After a second water rinse, place the slides in the second stain, aqueous hematoxylin, and let them stand for approximately 10 minutes.
  - The length of time required to stain sections with hematoxylin is extremely variable and, in general, can only be judged after slides are run through to Hemo-De®.
  - Remove excess hematoxylin with a water rinse and destain them in 4% aqueous ferric chloride for approximately 3-5 seconds.
  - Run the slides through three water rinses, adding a squirt of 1 M sodium hydroxide (NaOH) to the last rinse to make the hematoxylin stain slightly blue.
  - Hydrate the sections by placing the slides in a series of 50, 95, and 100% ethanol before placing them in Hemo-De®.
  - Following completion of the Hemo-De® step, view the slides under a light microscope to judge their appearance and assess the contrast of the stains.
    - If necessary, remove excess stain by running the slides backward through the dehydration series and destain further.
    - If tissues are not stained darkly enough, again run the slides backward through the dehydration series and correct the stain.
    - If sections show tissue browning, fresh hematoxylin should be prepared. These sections must be discarded.
  - When the desired stain has been achieved, rinse the slides in two to three changes of 100% alcohol to remove excess stain and to ensure complete dehydration.
  - Clear the slides with Hemo-De®.

2.11 Mounting the Stained Slide

2.11.1 Theory

The final operation in preparing microscope slides is to cement a coverslip on the preparation (1). Coverslips should be clean and free from moisture (1). To prevent damaging sections, place the coverslip exactly (4). Mounting media are chosen for their strength and keeping quality (1). Although balsam has been the most widely used medium and may remain in perfect condition for up to 25 years, it is prone to yellowing and cracking (1). Various synthetic resins, such as Histoclad® (resins in toluene), have been developed to improve the quality and longevity of slide preparations (1).

Choose cover glass size so that at least a 5-mm margin will exist between the stained sections and the edge of the cover glass (1).

2.11.2 Method

- Mount stained sections in Histoclad® (resins in toluene), or another suitable synthetic medium, to enhance the keeping quality of the slide.
  - Remove slides from the Hemo-De® clearing solution and place them tissue-side up on blotting paper.
  - Drain excess Hemo-De® from around the sections and place a drop of Histoclad® on the tissues.
  - Lower the selected cover glass slowly and obliquely onto the slide to prevent air bubbles.
  - Complete the mounting procedure as quickly as possible to prevent tissue desiccation.
  - Newly mounted slides may be dried in a 53°C oven for 24-48 hours to aid in hardening the mounting medium. Slides may also require further cleaning if they are filmy.
  - Store completed slides in a slide box to prevent dust deposits.

3 PROCESSING MATERIAL FOR SCANNING ELECTRON MICROSCOPE ANALYSIS

3.1 Introduction

Scanning electron microscopy is an excellent adjunct to conventional microscopy in studying various types of plant material. The main advantage of the scanning electron microscope (SEM) is its ability to produce images of solid specimens (1). Because of the significant increase in depth of field of scanning electron microscopy over that of light microscopy, images produced exhibit a striking three-dimensional accuracy (1). Procedures involved in preparing samples for scanning electron microscopy are different from those used in preparing material for paraffin sectioning and observation.

3.2 Dissection and Fixation

3.2.1 Theory

Material prepared for SEM analysis falls into three main categories:

1. stable and dry material, such as pollen or bark;
2. stable and moist material which includes samples suspended in aqueous solutions, such as bacteria in a drop of water; and
3. labile substances, such as shoot apices (1).

The majority of materials prepared for SEM study are labile and require delicate handling and processing (1). Plant material prepared for SEM observation must be as small as possible, fresh, and free from mechanical damage (1).

In general, samples should not exceed 2-3 mm^3. This standard is maintained to ensure complete fixation. Material dissected for SEM study must be as fresh and as hydrated as possible. Immediately following dissection samples should be scanned, or placed in the appropriate fixative to prevent damage caused by tissue desiccation. Dissection standards should be raised because it is important to remember that even slight damage to tissues during dissection becomes a significant problem when magnified over 10 000 times.

Fixatives used in preparing SEM samples differ from those used in procedures such as the paraffin method in that they penetrate slowly (1). However, because only the surface of the material is examined, fixation of internal structures is of lesser importance (1). Zirkle's solution is an example of an SEM fixative which preserves all cell structures well (1). Because this fixative contains toxic reagents it should be prepared in a fume hood and all handling should be accompanied by the use of protective lab wear (J.E. MacDonald, pers. comm., June 1988, University of Victoria, Victoria, B.C.).

3.2.2 Method

- Remove needles surrounding the apical bud by carefully stripping them away from the stem.
  - All needles on the upper 1.0-1.5 cm of the shoot should be removed.
  - Do not cut the apical bud from the shoot. Instead, keep the bud intact to help prevent tissue desiccation during dissection.
- Rapidly remove the bud scales by grasping them at the base with extra fine point forceps and detaching them from the bud.
  - If bud scales are removed correctly, the dissected vegetative shoot tip will be free from torn remnants of bud scale tissue.

When all bud scales surrounding the vegetative shoot tip have been removed, make a cross-sectional cut 2-3 mm below the base of the apical tissue to remove the vegetative shoot tip from the stem.

- Dissected material should not exceed 2-3 mm^3.

- Immediately following dissection, transfer the specimen to a vial containing Zirkle's SEM fixative (see Appendix 1, part B, section 1.0 for formulation, and Appendix 2, part A, section 7.0 for chemical costs), and attach a pencilled identification tag listing date, experimental treatment (if any), plant species, plant part fixed, and fixative used.

  Cork the vials and store them in a cool, dark area for further processing.

3.3 Post-fixation Tissue Preparation

3.3.1 Theory

Following fixation, specimens will store until final processing, which includes critical point drying, coating the samples with carbon and gold palladium, and photographing them under the SEM (1). While dissection and fixation can be completed with limited facilities, post-fixation processing must be done in a fully equipped SEM laboratory.

Fixed plant specimens must be completely dried before they can be observed using the SEM (1). To prevent tissue distortion caused by normal evaporative drying, samples are dried using the critical point drying method (1). In this procedure, the liquid in which the specimen is immersed, known as an intermediate fluid, changes to vapour without forming a liquid-vapour interface at the surface of the specimen, thus eliminating surface pressure (1). The liquid-vapour interface in the specimen is eliminated by heating the intermediate fluid in the tissues to a critical temperature within a closed chamber (1). The point at which liquid and gas densities are equal, termed the critical pressure, is the point of interface elimination within the tissues (1). Provided the samples are kept above critical temperature, the tissues can be completely bled without recondensation or distortion occurring. This way they maintain the natural physical appearance of the sample (1).

When samples have been completely dried they are mounted on prepared SEM specimen stubs (1). Samples are then coated with carbon and gold palladium in a vacuum evaporator (1). The completed specimen stub is then ready for examination and photography using the SEM (1).

3.3.2 Method

- Following fixation in Zirkle's solution, vegetative shoot tip samples are run through a tissue-processing schedule shown in Table 3. Transition of the material from dehydrating agent to intermediate fluid prepares the samples for critical point drying. (For costs of chemicals used in post-fixation tissue preparation see Appendix 2, part A, section 8.0).

  Run washed samples through the listed alcohol series to 100% ethanol.

  - Following dehydration, make the transition from the dehydrating agent to the intermediate fluid, amyl acetate.

  - Place the samples in solutions containing progressively higher parts of amyl acetate to 100% ethanol as outlined in Table 3.

- Before samples are put through critical point drying, soak them in 100% amyl acetate for 1 hour.

  - Freon and carbon dioxide are also commonly used as transitional fluids.

- Place the samples in the specimen chamber of a critical point dryer and process them until the tissues are completely dried.

- Using fine-point forceps under a stereomicroscope, arrange dried vegetative shoot tips at the periphery of prepared SEM stubs.

- Place the samples in a vacuum evaporator and coat the specimens with carbon and gold palladium. The specimens are now ready for scanning electron microscope observation.

TABLE 3. Post-fixation tissue processing schedule to prepare plant specimens for scanning electron microscope examination (J.E. MacDonald, pers. comm., June 1988, University of Victoria, Victoria, B.C.).

Step	Procedure	Reagent(s)	Time
1.	Rinse fixed material (several changes)	distilled water	2 h
2.	Run dehydration series	a) 10% ethanol	30 min
		b) 20%	"
		c) 30%	"
		d) 40%	"
		e) 50%	"
		f) 60%	"
		g) 70%	"
		h) 80%	"
		i) 95%	"
		j) 100%	"
		k) 100%	"
3.	Dehydration/intermediate fluid series	100% etOH : amyl acetate	
		a) 3 parts : 1 part	30 min
		b) 2 parts : 2 parts	"
		c) 1 part: 3 parts	"
4.	Intermediate fluid rinse	100% amyl acetate	30 min
5.	Critical point dry until bled		
6.	Tape specimens to prepared SEM stubs		
7.	Coat specimens with carbon/gold palladium in vacuum evaporator		
8.	Place stub in SEM holder and view		

4 LITERATURE CITED

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APPENDIX 1: Preparation of solutions used in processing plant material for paraffin sectioning and SEM observation.

All processing solutions should be prepared and used in the fume hood. Protective labwear including labcoat, protective glasses, and gloves must be worn at all times.

Section A. Solutions used in the paraffin method of processing tissue.

1.0 Fixation

a) Formalin acetic alcohol (FAA) (1)

Solution (i)	95% ethanol	352 mL
	formalin	48 mL
Solution (ii)	glacial acetic acid (1%)	40 mL
	distilled water	360 mL

Prepare solution (i) and (ii) in separate flasks. Mix in a 1:1 ratio immediately before using.

2.0 Dehydration

a) Johansen dehydration series (see Table A)

b) Alcohol dilutions

e.g. (i)	50% ethanol	
	95% ethanol	50 mL
	distilled water	45 mL

sample calculation:

$$100 \times \frac{\text{mL 95\% ethanol} \times 0.95}{\text{mL ethanol} + \text{mL } dH_2O} = \%\ \text{ethanol}$$

$$100 \times \frac{50\ \text{mL} \times 0.95}{50\ \text{mL} + 45\ \text{mL}} = 50\%\ \text{ethanol}$$

3.0 Softening

a) Gifford's solution[1]

glacial acetic acid (1%)	80 mL
60% ethanol	320 mL
glycerine (saturate)	20 mL

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[1] Biology 344 (Plant Microtechnique) Laboratory Manual (John Owens, compiler). Biology Dept., University of Victoria, Victoria, B.C. Unpublished.

TABLE A. Formulations of solutions used in the Johansen tertiary butyl alcohol (TBA) dehydration series of the paraffin method (4)

Solution number	Amount of solution added (mL)				
	dH_2O	95% ethanol	TBA	100% ethanol	paraffin oil
1	200	160	40	—	—
2	120	200	80	—	—
3	60	200	140	—	—
4	—	180	220	—	—
5	—	—	300	100	—
6	—	—	400	—	—
7	—	—	200	—	200

4.0 Mounting

a) Haupt's adhesive

knox gelatin	1 g
distilled water	100 mL
phenol crystals	2 g
glycerin	15 mL

Dissolve gelatin in distilled water at 30°C. After the gelatin is completely dissolved, add phenol crystals and glycerine. Filter and store in a dropper bottle.

5.0 Staining

a) Safrinin O 1% (aq)

safrinin O	1 g
distilled water	100 mL

b) Hematoxylin (Heidenhain's)

hematoxylin	2.5 g
sodium iodate	0.3 g
distilled water	500 mL

c) Ferric chloride 4% (aq) mordant

ferric chloride (anhydrous)	20 g
distilled water	500 mL

Do not shake the mordant after it is prepared. This may cause the ferric chloride to come out of solution.

d) Sodium hydroxide – 1M

sodium hydroxide	40 g
distilled water	1000 mL

Section B. Solutions used in preparing samples for SEM observation.

1.0 Fixation

a) Zirkle's SEM fixative

potassium dichromate	1.25	g
ammonium dichromate	1.25	g
cupric sulphate	1.0	g
distilled water	400	mL

Prepare Zirkle's solution on a stir plate in a fume hood.

APPENDIX 2: Cost breakdown of microtechnique materials based on 1988 cost estimates

Section A. Chemicals

1.0 Fixation – Formalin Acetic Alcohol (FAA)

a) Formalin (4 L)	$ 60.00
b) Glacial acetic acid (2.5 L)	25.00
c) Ethanol 95% (4 L) X 2	50.00
	135.00

2.0 Dehydration – Johansen TBA dehydration series

a) Tertiary-butanol (1 L)	25.00
b) Ethanol 100% (1 L)	30.00
	55.00

3.0 Infiltration/Embedding

a) Paraffin oil (1 L)	25.00
b) Tissue Prep® embedding medium (4 X 2 kg)	100.00
	125.00

4.0 Softening – Gifford's solution

a) Glacial acetic acid (2.5 L) as per section 1(b)

5.0 Mounting – Haupt's adhesive

a) Formalin (4 L) as per section 1(a)	
b) Phenol crystals (100 g)	45.00
c) Toluene (1 L)	20.00
	65.00

6.0 Staining – Safrinin O – Hematoxylin anatomy stain

a) Hemo-De® (1 gal.)	40.00
b) Safrinin O (10 g)	25.00
c) Ferric chloride anhydrous (100 g)	15.00
d) Hematoxylin (10 g)	30.00
e) Sodium iodate (100 g)	30.00
f) Sodium hydroxide (500 g)	15.00
	155.00

7.0 SEM fixative – Zirkle's solution

a) Potassium dichromate (100 g)	15.00
b) Ammonium dichromate (100 g)	15.00
c) Cupric sulphate (100 g)	10.00
	40.00

8.0 Post-fixation tissue preparation – SEM

a) Ethanol 100% (1 L) as per section 2(b)	
b) Amyl acetate (500 mL)	25.00
Total all chemicals	~ $ 600.00

Section B. Labware

1)	Boston round bottles (500 mL) X 24	$ 40.00
2)	Staining trays	200.00
3)	Dropping bottles X 2	10.00
4)	Microscope slides X 72	5.00
5)	Cover slips X 230	10.00
6)	Metal putty knife	6.00
7)	Wash bottles X 2	5.00
8)	Pipettes (10 mL) X 10	10.00
9)	Pipetter (10 mL)	20.00
10)	Fine point forceps	4.00
11)	Glass vials (21 mm X 70 mm) X 144	50.00
12)	Corks #8 X 100	15.00
13)	Latex gloves X 100	25.00
Labware total		≈ $ 400.00

Section C. Lab Equipment

1.0 Embedding

a) Warming plate (e.g., Eberbach 2750) $ 200.00

2.0 Sectioning

a) Rotary microtome (e.g., AO Reichert [AO820C]) 3500.00

3.0 Mounting

a) Slide warmer (e.g., Precision Sci. 66632) 600.00

Lab equipment total ≈ $ 4300.00

A.	Chemicals	600.00
B.	Labware	400.00
C.	Lab equipment	4300.00
Total all materials		≈ $ 5300.00